Genes, guts and globalisation

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THE 2017 GARROD LECTURE: GENES, GUTS AND GLOBALISATION

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ABSTRACT

The widespread use of antibacterial drugs over the last 70 years has brought immense benefits to human health at the price of increasing drug inefficacy. Antibacterial agents have a strong selective effect in both favouring resistant strains and allowing particular species and families of bacteria to prosper, especially in the healthcare setting. Whilst important gram positive bacterial pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae* caused concern over the last 20 years because of the spread of antibiotic resistant strains, Enterobacteriacae have become the biggest challenge. They have very efficient mechanisms for genetic exchange as illustrated by the emergence and rapid spread of CTX-M beta-lactamases and the carbapenemases. The unique epidemiology of Enterobacteriacae with substantial numbers colonising the mammalian gut and subsequent release into and spread in the environment presents a significant threat to human health because of the high levels of exposure for the whole community. The use of antimicrobials in agriculture combined with global movements of people, animals and food arising from worldwide industrialisation generates a diversity and level of resistance not seen previously. Control will require globally co-ordinated interventions similar to those needed to ameliorate climate change.

INTRODUCTION

Professor L.P. Garrod, in whose honour this lecture was inaugurated in 1982 by the British Society for Antimicrobial Chemotherapy, was a pioneer of linking laboratory investigation to the clinical outcome of antimicrobial chemotherapy. In 1954 Garrod published the results of the first comprehensive clinical trial of antimicrobial susceptibility testing and clinical outcome in urinary tract infection. He made the observation that outcome was closely linked to the in vitro antibiotic susceptibility of individual strains of different bacterial species in the routine laboratory[1]. He can, therefore, be regarded as one of the first to study the epidemiology of antimicrobial resistance in Enterobacteriacae, the subject of this Garrod lecture.

I feel very privileged to have been invited to deliver this Garrod lecture for 2017, particularly as I attended the first lecture given by Professor Sir Mark Richmond in 1982 at the Zoological Society of London. He, together with Professor Peter Bennett and Professor David Speller, supervised my MD degree at the University of Bristol in the early 1980s. The project focused on gentamicin resistant *Providencia stuartii* that were causing infections particularly in Care of the Elderly Wards (CoEW). The reservoirs and mode of spread of *P.stuartii* were poorly understood, as clusters of cases occurred with colonised urine in catheters as the presumed source of infection, at other times occurrence of overt infection was sporadic with no apparent source[2] . A detailed study of a male CoEW showed that faecal colonisation represented the most significant reservoir [3]. During the course of collecting strains for this study a series of isolates were obtained over a 20 month period from a patient with a long term urinary catheter colonised by a single serotype (O:63) of *P.stuartii.* The isolates suddenly developed resistance to carbenicillin having previously been susceptible. Plasmid isolation, restriction endonuclease digestion mapping and isoelectric focussing of the β-lactamase from these resistant isolates showed that a 34 kilobase cryptic plasmid carried by the sensitive isolates had acquired two copies of the class II transposon TnI encoding the TEM-2 β-lactamase [4]. This turned out to be an extremely rare example of horizontal transfer of antibiotic resistance genes in the clinical environment. Such transfer events are key to the

evolution of antibiotic resistant plasmids and although presumed to occur quite frequently, are hardly ever observed in nature. The class II transposable element IS 1071, has been hypothesized to be involved in multiple mobilisations of a number of the important polychlorinated biphenyl (PCB) catabolizing genes found in environmental bacteria in industrially polluted soils. The remarkable similarity of the flanking IS 1071 sequences, despite huge geographic separations, suggested the same process had occurred as found in my *P.stuartii* strains[5]. My work at Bristol sparked a lifelong interest in the evolution of the genes and mobile elements encoding β-lactamases and the dynamics of faecal carriage of the host bacterial species.

EPIDEMIOLOGY OF β-LACTAMASE GENES

The ability of antibiotic resistant *Shigella* spp to transfer specific resistance markers to *E.coli* by cell to cell contact (conjugation) was first recognised by the Japanese scientists Watanabe, Mitsuhashi and Akiba between 1959 and 1961[6]. Naomi Datta, working at The Royal Postgraduate Medical School, Hammersmith London, produced the first report of conjugative antibiotic resistance transfer in Europe reporting transfer of streptomycin, tetracycline and sulphathiazole resistance between *Salmonella typhimurium* and *Shigella sonnei*[7]. She went on to identify a β-lactamase produced by an isolate of *Escherichia coli* from the blood culture of a patient in Greece named Temionera, so the enzyme was named TEM. This was the first β-lactamase from Gram negative bacteria identified to be carried on a transmissible R factor (plasmid) and to be purified in scale and characterised, its ability to hydrolyse the recently introduced antibiotic ampicillin was particularly significant [8]. The genes encoding the TEM β-lactamase were subsequently shown to be capable of replicative transfer from one plasmid (the early R plasmid RP4) to another and was the first antibiotic resistance transposon to be identified [9]. Transposition is defined as the insertion of a DNA sequence into a new location, on the same molecule or on a different one, ie a DNA rearrangement by a specialised form of recombination ,that is are able to transpose independently of the rest of the structure. Various types of transposon exist, the most “evolved” being the complex transposons. The genes concerned with transposition and other unrelated genes

are located side by side and form a single mobile module. Transposons are defined as transposable elements that accommodate at least one gene unconnected with transposition the expression of which confers a predictable phenotype on the cell, for example an antibiotic resistance gene in the case of antibiotic resistance transposons. These elements may be carried on the bacterial chromosome, or by a bacteriophage or a plasmid. The most common location for antibiotic resistance transposons is on a conjugative plasmid. Whether this reflects a natural distribution or experimental bias is not clear, but whole genome sequencing and metagenomics studies should provide an answer to this question the future.

A further mechanism for the movement of antibiotic resistance genes was recognised about 20 years ago namely the integron. Integrons are site specific recombination elements which mediate the organised transfer of genes on cassettes [10]. Antibiotic resistance genes are the most commonly carried genes and therefore represent an important molecular mechanism for horizontal gene transfer. The integrons carry their cassettes downstream of a recombinase encoding a conserved sequence (CS) which carries a strong promoter [11]. This promoter enables the expression of the inserted cassettes which can have originated from unrelated bacteria which use different sigma factors to the new host bacterium. Integrons are found on a wide range of conjugative plasmids some of which have a remarkably wide host range. It is striking that the metallo-carbapenemases IMP and VIM are almost exclusively found on class I integrons.

During the 1960s the widespread use of the aminopenicillins such as ampicillin and carbenicillin together with other antibiotics such as tetracyclines, chloramphenicol and sulphonamides drove increasing rates of resistance particularly as they are all mediated by R factors. The extent of this resistance, its strong association with transmissible plasmids and its widespread distribution in the faecal flora of the general population was identified by a seminal study by Naomi Datta published in 1969[12] . Patients admitted to the Hammersmith Hospital, London for routine surgical procedures were asked to provide a sample of faeces on admission, following their operation and before leaving hospital. Antibiotic resistant Gram negative bacilli were isolated on MacConkey's agar formally identified and tested for susceptibility to ampicillin, streptomycin, tetracycline, chloramphenicol, kanamycin, sulphathiazole, polymyxin B, nitrofurantoin and nalidixic acid. The ability of these resistances to

transfer to *E.coli* K12 was then determined. Seventy of the 100 pre-admission specimens yielded resistant coliform bacteria of which 52 were resistant *E.coli*. The majority (61%) of the 139 resistant strains of *E.coli* on admission, following surgery and before discharge transferred resistance to *E.coli* K12. There was only a slight increase in resistance rates in samples taken during hospital admission when compared to the rates in the community. Sulphonamide and tetracycline resistance was common with associated streptomycin resistance in a smaller number of strains. Ampicillin resistance was by the time of the survey (1968) already common in the community *E.coli* as 18/81 (22%) resistant *E.coli* pre-admission were resistant to ampicillin and therefore must be presumed to have been carrying the TEM β-lactamase. The difficulty of predicting susceptibility and the importance of susceptibility testing was emphasised in the discussion. The following chillingly accurate prediction was made "drugs to which coliform bacilli are still usually sensitive such as kanamycin or gentamicin must be brought into use, which will in turn have the effect of favouring resistant bacteria and may result in the dissemination of R factors conferring resistance to increasing numbers of drugs" [12]. Indeed in the latter part of the 1970s nosocomial outbreaks of cross infection by gentamicin resistant *Klebsiella pneumoniae* were reported, one of the earliest coming from Bristol, UK [13]. They identified asymptomatic faecal colonisation of patients as the source of infections caused by the *K. pneumoniae* with secondary colonisation of patients' skin and transmission on staff hands as the route of spread. Introducing routine faecal screening followed by patient isolation completely controlled the outbreak.

Investigation of the mechanisms of resistance to gentamicin in these outbreaks identified aminoglycoside inactivating enzymes to be responsible, the genes often residing on transposons located on plasmids. The first study to really conclusively demonstrate the movement of a common plasmid amongst different bacterial species over time using molecular methods was that undertaken in a hospital in Boston, USA by Tom O'Brien and colleagues[14] . They followed an outbreak of gentamicin resistant Gram negative bacilli which occurred initially in a single strain of *K.pneumoniae* which carried plasmid mediated resistance to gentamicin due to 2" aminoglycoside nucleotidyltransferase – AAD (2") as well as resistance to sulphonamides, chloramphenicol and the production of TEM 1 β-lactamase

conferring resistance to ampicillin. Between 1975 and 1977 this Inc M plasmid which had a highly conserved *Eco* RI DNA restriction endonuclease digestion pattern spread to multiple species of Enterobacteriacae and finally became dominant in *Serratia marcescens* and *E.coli.* The authors commented "These observations emphasized that resistance genes are too complex to arise often by chance mutation, so most strains of bacteria have to get them by transfer from other strains. Thus the delivery of an antibiotic resistance gene through the world's bacterial flora to a strain exposed to that antibiotic may be as necessary for the emergence of resistance as is the antibiotic exposure."[14] .

Although this paper was written nearly 40 years ago the same basic principles apply to the scientific community' efforts to counter the spread of resistance to the latest antibiotics such as carbapenems and the diaza-bicyclo-octane β lactamase inhibitors. It is also critical to understand the epidemiology of the host bacterium and then to investigate the movement and evolution of plasmids within those bacterial hosts and then the movement of individual antibiotic resistance genes between plasmids and the chromosome. Without an understanding of each one of these aspects it is not possible to truly characterise the movement of antibiotic resistance genes in humans, animals or the environment.

At the beginning of the 1980s the prospects for reliably treating serious infections caused by Enterobacteriacae were poor. However, the development of the extended spectrum cephalosporins, more frequently referred to as third generation cephalosporins (3GCs), starting with cefotaxime followed by ceftazidime and ceftriaxone saved the day. These antibiotics were stable to almost all the then circulating β lactamases, particularly those found in nosocomial strains of *Klebsiella, Enterobacter* and *Serratia* and therefore almost all the other Enterobacteriacae were susceptible to these new antibiotics.

It would not be long before bacterial evolution put bacteria ahead in the eternal arms race that is antimicrobial treatment. In France, where cefotaxime had been heavily used since 1980, strains of *K.pneumoniae* were reported in 1987 from isolates collected as early as 1984 which were particularly resistant to cefotaxime were found to produce a plasmid encoded β lactamase then called CTX-1 [15]. The β lactamase

was then recognised as being derived by mutation from the widely distributed TEM-2 β lactamase (not capable of degrading 3CGs and very closely related to TEM-1) by two amino acid substitutions of which the G238S conferred a substantial increase in the hydrolysis of the cefotaxime and ceftazidime [1617].

A number of different combinations of mutated TEM and SHV derived β lactamases were discovered in short succession that were capable of hydrolysing 3GCs, giving rise to the term extended spectrum β lactamases (ESBLs)[18] . There have been a number of definitions of ESBL. The most pragmatic is: "a β lactamase, generally acquired rather than inherent to a species, that is either able to confer resistance to oxyimino-cephalosporins (but not carbapenems) or that has had an increased ability to do so, as compared with classic numbers of its genetic family (ie a mutant)" [19]. In order to be meaningful the definition requires that the ESBL class also be specified, eg TEM ESBL or CTX-M ESBL. As the number of both individual genotypes of ESBL and molecular families expanded it became clear that a range of MICs to 3GCs existed some of which were found to be in the susceptible range. This led EUCAST and CLSI to recommend that susceptibility results should be reported "as found" and the presence of either ESBL genes or the phenotypic characteristics of ESBL not be sought. An expert group convincingly argued that ESBLs should continue to be sought for three reasons. Firstly although there are cases where 3GCs have been effective in treating infections due to ESBL producers that exhibit a low MIC, there are a similar number of cases that failed treatment. Secondly routine susceptibility testing is less accurate than research methods and ESBL producers with MICs 1-8 mg/L will oscillate between susceptible and resistant depending on who tests them. Finally, although EUCAST and CLSI advocate testing for epidemiological purposes, it is likely that many laboratories will not test at all leading to a loss of critical information. It is, therefore, prudent to continue to search for ESBLs among all isolates and when found generally to avoid substrate drugs for therapy [20]. Retrospective analysis of a strain of *K.oxytoca* isolated in 1982 from a neonatal unit in Liverpool, England, represents the earliest known isolate of ESBL in the UK [21]. There had been an outbreak of nosocomial infection due to a gentamicin resistant but ceftazidime susceptible strain of *K.oxytoca* that produced TEM-1 β lactamase. Patients were treated with ceftazidime but subsequent isolates were resistant to ceftazidime. DNA sequencing showed the mutant TEM-1 gene was

carried on a 141 kb plasmid in all the isolates with a G164S mutation associated with the ESBL phenotype. This genotype is now recognised at TEM-12[21] .

The TEM ESBL emerged worldwide and caused a number of outbreaks [2218]. Some particular genotypes such as TEM-10, 12 and 26 pertained particularly common in both Europe and North America and these all carry a mutation R164S which has been shown to further enhance hydrolysis of ceftazidime over the original TEM-3 G238S mutation[17] . The evolutionary value in clinical settings of these mutations conferring the ESBL phenotype was brought to my attention when investigating an outbreak of cefotaxime resistant Enterobacteriacae on a paediatric oncology ward at St James' Hospital, Leeds, England between November 1989 and January 1990 [23]. A total of 81 isolates of 6 species of Enterobacteriacae (*K.oxytocoa* and *E.coli* were the most common with 28 isolates of each species) were analysed in detail revealing the presence of TEM-10B, TEM-12B and TEM-26B carried on plasmids ranging in size form 2.5-150 kb. This was a complex outbreak with some patients carrying multiple species containing mixtures of TEM genotypes which was probably driven by the fact that we had shown that at least the bla_{TEM-12} genes were transposable[24] . We then realised that our TEM genotypes were derived from TEM-1, whereas the TEM-10, TEM-12 and TEM-26 reported from the USA were derived from TEM-2 [25].This represents one of the few characterised examples of convergent evolution by selection from different antibiotic resistance genes leading to common genotype. Furthermore we observed identical ribotype strains of both *E.coli* and *Klebsiella* producing both TEM-12B and TEM-26B suggesting that the single point mutation had been acquired on the ward[23]. We produced evolutionary trees to suggest the likely paths of evolution by mutation of the genes taking account of synonymous and non-synonymous nucleotide changes. TEM-26 has a single mutation E104K (glutamic acid to lysine) which is now recognised to increase ceftazidime hydrolysis approximately 40-fold[17] suggesting it would be favoured in a clinical environments with heavy usage of ceftazidime. It is tempting to speculate that the early TEM-12 producing *K.oxytoca* found in 1982 in Liverpool had survived and spread in the north of England undetected and seeded the Leeds outbreak seven years later.

During the 1990s it seemed that TEM and SHV ESBLs were not spreading widely and did not appear to be producing catastrophic levels of resistance to 3GCs. They also did not seem to spread widely into the environmental and commensal pool of Enterobacteriacae, even in countries where ESBL phenotype rates were being reported as high such as China and India although there were very little data from those countries. The world was on the verge of experiencing arguably one of the biggest, most serious antibiotic resistance outbreaks, namely the emergence from an environmental bacterium *Kluyvera* and the global spread of the CTX-M family of ESBLs. The event has been justifiably described as a pandemic [26].

THE ORIGIN AND EVOLUTION OF THE CTX-M PANDEMIC

. The initially named MEN-1 ESBL from an *E.coli* isolate characterised in France from a patient in Italy (1990) was found to be identical to CTX-M-1 from an *E.coli* isolated from a child in Germany (1989)[27]. The same paper also reported 2 further isolates of CTX-M-1 producing *E.coli* from Germany in 1994, but they were regarded as examples of unusual β-lactamases because of their rarity in Europe. In the 1990s in South America the emergence and recognition of CTX-M-2 was seen as much more serious as the enzyme was found in *Salmonella typhimurium, E.coli, Klebsiella* spp. and *Proteus mirabilis* in Argentina and Paraguay [28]. We now realise this was a turning point in the evolutionary history of β-lactamases in *Enterobacteriacae.* In the case of the CTX-M gene it emerged in *Enterobacteriacae* as a result of horizontal gene transfer from an environmental bacterium living in the rhizosphere. The chromosomal homologs of the CTX-M genes in different species of *Kluyvera* have been mobilised into *Klebsiella* spp and *E.coli* on different occasions and global locations, which gave rise to 4 sub lineages often referred to as “groups” (CTX-M-1,2,9,&8; named after the archetypal enzymes of each group). A phylogenetic analysis suggested that the mobilization from *Kluyvera* spp chromosomes happened twice for the CTX-M-2 group, at least 3 times for CTX-M-1 whereas, CTX-M-9, CTX-M-8 and CTX-M-25 groups all only mobilised once[29]. The indisputable proof of this evolutionary route came from work in Paris by Poirel and Naas that showed that the neighbouring sequences of *bla* $_{\text{CTX-M-2}}$, *bla* $_{\text{CTX-M-5}}$ and Toho-1 were identical to those in the flanking chromosomal sequences in *Kluyvera ascorbata* [30]. *Kluyvera* spp are

one of a number of species of bacteria that solubilize inorganic phosphate to make it available to plants [31]. They form part of the complex plant microbiome which is located in the root mycorrhizae which are comprised of a very large number of bacteria and fungi including the Streptomycetes and Actinomyctes bacteria many of which produce antimicrobial metabolites[32]. These bacteria are used to produce many of our therapeutic antibiotics. *Kluyvera ascorbata* has been shown to produce a siderophore which allows plants to grow in the presence of nickel contamination. Using fluorescence staining *K.ascorbata* has been showed to be tightly attached to roots and seeds of many plants [33]. It is entirely plausible that *Kluyvera*.*spp* produce an inducible broad spectrum β lactamase to facilitate their survival in the mycorrhiza. All the species of *Kluyvera* have been noted to have chromosomal copies of an inducible enzyme almost identical to the plasmid mediated CTX-M β lactamases.[30] . Subsequently work in China showed that another CTX-M variant, CTX-M-14,was more common than SHV and TEM type ESBLs, with smaller numbers of CTX-M-3 [34]. Simultaneously CTX-M-15 was described in Paris from six of isolates from New Delhi in India. These two particular genotypes have assumed worldwide dominance with reports from studies in India and China showing these to be the dominant CTX-M, and in the case of India CTX-M-15 being the only genotype present in the entire country[3536] .

My involvement with the CTX-M β-lactamases dates from my first visit to China in 1998 to run a course on the detection of antibiotic resistant bacteria for the WHO Emerging Pathogens Initiative at the First Municipal Peoples Hospital in Guangzhou. Dr Jian Hui Xiong had carried out a survey of resistance of Gram negative bacilli to various antibiotics using NCCLS methodology and approved media. The ESBL production rate was 33% for *E.coli* and 37% for *Klebsiella pneumoniae*. This was an exceptional finding so molecular characterisation was undertaken in my laboratory in Leeds.. There had only been a single confirmed report of SHV-2 in *Klebsiella spp.* and *Enterobacter* spp. in 1994 [37] A total of 15 isolates of *Enterobacteriacae* including 8 *E.coli* and 3 *K.pneumoniae* from Guangzhou were fully characterised. SHV ESBL genes (SHV-11 & SHV-12) were found in 8 isolates but none of the other commonly recognised ESBL genes, were found using PCR. I thought this may be a rare ESBL gene so in view of the strong cefotaximase activity a number of consensus PCR primers for various rarer ESBL genes including CTX-M were designed. We were

delighted to obtain product from the PCR primers for CTX-M for 13 of the 15 isolates.[34]**.**

Subsequent transfer of plasmids and full sequencing with cloning revealed 3 novel CTX-M genes for which we were allocated the genotype numbers 13,14 and 15. Whilst the paper was in proof it was pointed out that our CTX-M-15 was identical to CTX-M-9 reported by Sabate and colleagues in Spain. We had carefully checked our sequence and it was one base different to that in in Genbank for CTX-M-9. However, when we rechecked it in Genbank it appeared that the entry had subsequently been re-edited to the same sequence that we had, hence we amended our paper to refer to CTX-M-9 rather than CTX-M-15. Ironically the number went back into the pool and was allocated to a *bla* $_{\text{CTX-M}}$ gene found in some Indian isolates characterised in Paris which is now recognised as the most common *bla* $_{\text{CTX-M}}$ worldwide[38] .

Our identification of CTX-M-14 was the first description of what subsequently became the world's second most common CTX-M ESBL. Most importantly we identified ISE*cp1*as the likely mobilising element for *bla*$_{\text{CTX-M-14}}$ as I knew it had been reported by P.D. Stapleton in an ICAAC abstract in 1999 as mobilising the chromosomal AmpC to become CMY-4 plasmid mediated AmpC β-lactamase [39].Interestingly ISEcp1 was also associated with the mobilisation of *bla*$_{\text{CTX-M15}}$[30]. Our Southern blots clearly showed the presence of the *bla* $_{\text{CTX-M-14}}$ gene on both the chromosome and plasmids suggesting mobilisation most probably by one ended transposition. We also demonstrated the migration at least in some strains to the chromosome of recipient strains following conjugation. This was the first description of the mobilisation of *bla* $_{\text{CTX-M}}$ between replicons. It was also the first study to reveal the extent of the problem of ESBLs in China and to show that CTX-M-14 as the foremost enzyme. The concluding sentence in our paper turned out to be prophetic: "the ease with which such bacteria can be isolated should be a cause for grave concern and indicates the need for more detailed surveillance and epidemiological surveys in this region, which has increasing contact with the rest of the world."[34]

Appearance and spread of CTX-M in UK

The appearance of CTX-M β lactamases in the UK occurred in a number of centres which perhaps supports the hypothesis of multiple introductions from the two areas of the world where CTX-M β lactamases were most common, i.e. India and Pakistan and China. A single isolate of *Klebsiella oxytoca* producing CTX-M 9 was found in a patient in Leeds in May 2000[40] . The first published UK outbreak was from Birmingham from between July 2001 and February 2002 involving 33 patients all infected with *K.pneumoniae*[41] . The start of the outbreak had been missed as a number of isolates of *K.pneumoniae* were noticed earlier in 2001 that were borderline susceptible to ceftazidime by disc diffusion. It was only when the isolates were tested to cefotaxime and cefopodoxine that MICs of 2.0mg/L were noted and the recommendation was made in the publication to test either of these agents to detect all CTX-M genotypes[41] . The genotype turned out to be the first description of CTM-X 26, which has only been reported from the Birmingham area and subsequently Israel[42].

Following the single report of CTX-M 9 isolated in May 2000 in Leeds [40] and the outbreak in Birmingham involving the clonal spread of a single strain of *K.pneumoniae* producing CTX-M 26 in 2001[41] there appeared the first published record of the occurrence of CTX-M-15 in the UK [43]. Prompted by our reports, The Health Protection Agency Antimicrobial Resistance Reference Laboratory examined a collection of isolates of Enterobacteriacae made in 2001 as part of a survey of susceptibility to piperacillin/tazobactam. Amongst 122 cephalosporin resistant isolates of Enterobacteriacae 7 exhibited cefotaxime MICs at least 8-fold greater than the ceftazidime MIC. These were selected and screened for the presence of bla_{CTX-M} and 4 isolates of *E.coli* were found to carry $bla_{CTX-M-15}$, 2 from one hospital in London, and one each from Newcastle-upon-Tyne and Belfast[43] . It was entirely possible that CTX-M ESBLs had been present for some time at low levels. However, they certainly were not present before 1991, as an examination of a collection of 3,951 non-duplicate isolates of Enterobacteriacae from 96 hospitals across the UK collected in 1990-1991 only identified five ESBL producing isolates which were all SHV ESBLs[44] . Subsequent screening of these isolates with CTX-M PCR primers failed to identify any bla_{CTX-M} genes (Hawkey unpublished data). The speed and diversity of genotypes with which CTX-M β-lactamases penetrated the UK resistome at this time was illustrated by a study of the faecal carriage of CTX-M

in the faecal flora of 1000 individual outpatients at York District Hospital. A total of 17 isolates of *E.coli*, *Enterobacter cloacae, Klebsiella* spp. *Citrobacter freundii* produced CTX-M β-lactamases (5 $bla_{CTX\text{-}M\text{-}15,}$ 3 $bla_{CTX\text{-}M\text{-}14,}$ 9 $bla_{CTX\text{-}M\text{-}9}$),[45] . In a study of the earlier isolates found at the Health Protection Agency and other isolates submitted to the Reference Laboratory at Colindale it was established that CTX-M-15 was the most frequently encountered genotype in the UK and that the earliest isolate in that collection was from May 2001[46] . This was challenged by a study from Bristol which reported a single isolate of *E.coli* producing CTX-M-15 from an abscess in an Indian lady made in May 2000 who had visited India in 1999[47]. However, results from one of our PhD students in Birmingham showed that CTX-M producing *E.coli* well established in Birmingham in 2000 which with a substantial population from South Asia suggests prior importation[48] . Nineteen isolates of *E.coli* producing CTX-M 15, all from the Queen Elizabeth Hospital, were made in 2000, the earliest being in July 2000, the majority (15) coming from ITU and Hepatology wards (see figure 1). RAPD strain typing showed 7 isolates to belonged to one strain and 5 to another distinct strain the rest were distinct strains. In 2001, 10 isolates of *E.coli* were made largely from the original wards but now including an associated hospital and the first community isolate. In 2002 the situation dramatically changed with 80 isolates being recovered from patients in all clinical specialties in the hospital and as well as 2 wards at a local orthopaedic hospital. Only 2 isolates were cultured from the patients on the Liver and General Intensive Care Unit which was the first ward affected by the outbreak in 2000. Significantly 9 isolates came from community patients suggesting the genes had now spread into the wider community. The genotype distribution became much more complex (see figure 1) with CTX-M-15 producing *Klebsiella* and *E.cloacae* being identified as well as CTX-M-14 and CTM-M 26 which had caused on outbreak in 2001 at City Hospital on the west side of Birmingham, some 8 miles away from the Queen Elizabeth Hospital.

CTX-M-15 is the dominant genotype in the UK which has been ascribed to its strong association with the pathogenic clone of *E.coli* O:25_b-ST131 following early recognition of this clone in an outbreak in Shrewsbury in 2003 in the West Midlands. The strain was designated strain A[49] and possessed the characteristics of the internationally dispersed *E.coli* phylogenetic group B2, serotype O:25 and sequence type ST131 recognised by Nicolas-Chanione and colleagues in 2007[50] . They

identified these clonally related strains of CTX-M positive multi drug resistant virulent *E.coli* clonal group with highly homogeneous virulent genotypes and subgroups exhibiting highly similar PFGE profiles suggesting its recent emergence. Three years after the initial study by Woodford in April-May 2006 we carried out a study of 294 clinically significant ESBL positive isolates of Enterobacteriacae from 11 hospitals across the West Midlands of which 232 were *E.coli* that produced CTX-M β-lactamases[51] . Two hundred and eighty four produced CTX-M-15, the remaining 10 isolates producing CTX-M-14(4), 9(2), 2(2) and 26(2). Whilst strain A (ST 131) was the dominant clones 30% of isolates producing CTX-M-15 were of different sequence types. This suggests there is a greater diversity of E.coli ESBLs producing infections in the UK than is often thought. Recently an analysis of 95 complete *E.coli* genomes of phylogroup B2 confirmed two earlier studies that North America was the likely location for the emergence of ST131 [52]. CTX-M-15 is particularly associated with Clade C2 (also referred to a H30-Rx) which is one of the 3 subclades identified in ST131 which are typically resistant to fluoroquinolones which have been suggested as a selective factor in the clade's success. The authors suggested that clade C diverged from B in about 1980 with the emergence of C2 in 1987. Whilst acquisition of fluoroquinolone resistance was associated with a global rise in the C2 clade it was the prior acquisition of a variety of virulence factors that was a prerequisite for its global success. Although acquisition of the CTX-M-15 gene carried on plasmids in C2, this property in itself does not explain the success of ST131 as the population expansion occurred in both C1 (lacks CTX-M-15) and C2. The group also showed that the plasmids that carry CTX-M-15 are typically diverse with considerable variation in their DNA sequences [52]. In an extensive study of the CTX-M ESBL plasmids in a broad ranging study of isolates of ST131 *E.coli* collected in the UK that carried CTX-M-15 the plasmids were found to be mainly of the IncFIA4 and Inc FIA1 groups which includes the originally described UK CTX-M-15 carrying plasmids which are similar to pEK499 [53]. The spread in the UK of $bla_{CTX\text{-}M\text{-}15}$ was therefore attributed not just to clonal expansion but also to the horizontal dissemination of related plasmids.

Global Distribution of Genotypes

There is a striking occurrence of particular genotypes in certain regions of the world e.g. CTX-M-14 in China, CTX-M-15 as the only genotype in India/Pakistan, CTX-M-3 in South America, CTX-M-1 in Poland, Russia and Italy/Libya. In a review this geographical clustering was summarised using a world map with pie charts representing the proportions of genotypes reported [54]. The hypothesis was advanced that an emergence in particular locations of CTX-M was favoured by high rates of antibiotic usage and poor health infrastructure (particularly sewage treatment).As the plasmid mediated CTX-M genes evolved by mobilisation from the chromosome of *Kluyvera* spp. Each mobilisation from a different species and geographical location gave rise to the locally prevalent genotype(s). The subsequent spread of those evolved genotypes was then via the movement of people, demonstrated by the same genotypes appearing in countries with clear cultural/historical connections e.g. India and UK; Italy and Libya. The widespread movement of the Chinese often carrying with them CTX-M-14 and people from South Asia(India ,Pakistan, Bangladesh and Sri Lanka) with CTX-M-15 seemed to be the driver for the global spread of these two globally dominant CTX-M genotypes. In addition to the first recognition of CTX-M as the dominant ESBL in China [34] and CTX-15 in India[35] we have undertaken genotyping surveys in other countries.

There were very few data from Arabian Gulf countries so we collaborated with microbiologists in Kuwait.[55] . The CTX-M-15 genotype was found to be totally dominant (27/29 isolates the remaining only two being CTX-M-9). This finding could be explained by the very large numbers of guest workers, largely from India and Pakistan. We also found CTX-M ESBLs to be more common in non- Kuwaiti Arabs with a history of recent travel. Although very close geographically and culturally to the UK the first genotyping study in Ireland demonstrated some significant differences between the two countries [56]**.** A total of 812 isolates of Enterobacteriacae from throughout Ireland were collected of which 506 from 462 patients harboured ESBL's. A single isolate from each patient was studied in more detail and all were subjected to PFGE analysis, a total of 371/462 being detected by our PCR method for CTX-M. Each PFGE type had a representative isolate genotyped by dHPLC for *bla* $_{\text{CTX-M}}$ genotype. *bla* $_{\text{CTX-M15}}$ was found in 177 isolates, *bla* $_{\text{CTX-M-14}}$ in 78 isolates and with 3 isolates harbouring *bla* $_{\text{CTX M-9}}$ and a single isolate *bla* $_{\text{CTX-M-1}}$. The occurrence of CTX-M-14 was much higher than in the UK which may reflect the

greater involvement of the population in beef and milk production. In England CTX-M-15 and CTX-M-14 are roughly equally distributed in cattle according to the UK Veterinary Laboratory Agency [57].

We have now updated the global genotype map in a recent review[58] . Much more data are now available and some interesting recent changes can be observed (Figure 2). China has previously been dominated by CTX-M-14 and CTX-M-9 since the first studies in the 1990s but in 2004/5 a study from Changsha in Hunan province CTX-M-15 emerged in 17.4% of CTX-M positive isolates presumable as a result of importation probably from South Asia[59] . A further study of Chinese CTX-M producing isolates from Changsha in 2013/14 showed that they now comprised 27.5% of isolates. They were mainly *E.coli* O:25bST131 but some carried the previously undescribed *fimH41* gene, which had presumably been acquired by recombination and has never been reported from this clade[60]. A single locus variant of CTX-M-15, CTX-M-55, has also become more common in China. The other significant shift in genotypes has been the rise of CTX-M-27, which originally was described in France in 2003 as a single locus variant of CTX-M-14 with a D240G mutation which enhanced ceftazidime hydrolysis [61]. It has been increasing in frequency in China, Japan, South East Asia, North America and Europe[58].

FAECAL CARRIAGE OF CTX-M PRODUCING BACTERIA

The natural habitat for many species of the Enterobacteriacae such as *E.coli* and *Klebsiella* spp. is the mammalian gut, so estimating the rates of colonization and antibiotic resistance is very important. This was encapsulated by the concluding remarks of Naomi Datta in one of her papers nearly 50 years ago. “Any measures which may be introduced to control or eliminate the spread of R factors will be assessable only if their incidence is followed over a period of years in normal intestinal bacteria as well as enteric pathogens” [12].

There are widely differing rates of CTX-M production in *E.coli* and *Klebsiella*, which is assumed to relate to the interplay between high levels of antibiotic use in man and animals and the lower availability of safe sewage disposable and clean drinking water in countries with high rates of ESBL carriage. Worldwide surveillance for resistance in Enterobacteriacae has been poor prior to the institution by the WHO of

the GLASS programme. The most comprehensive and established data comes from the SMART programme supported by Merck & Co. Inc which surveys the antibiotic susceptibility of Enterobacteriacae causing intra-abdominal infection. Data from the Asia Pacific region for 2008 showed that ESBL rates in *E.coli* were 61% India, 59% China, 53% Thailand, 12.3% Singapore and 2.9% Malaysia which supports this hypothesis[62]. Visitors to areas with high resistance rates acquire ESBLs and of the genotype dominant in that location, as was shown by a recent study that 75% of those traveling to South Asia acquired faecal carriage of ESBLs which were overwhelmingly of the CTX-M type [63]. Rates have been rising in all WHO regions since 2002 with the fastest growth occurring in the South East Asia and Eastern Mediterranean [64]. We investigated the rate of carriage of CTX-M producing *E.coli* in different sections in the community in Birmingham to ascertain what the degree of penetration of ESBLs was into the UK healthy population faecal resistome. We examined 732 faeces samples in 2010 from individuals in the community that had submitted samples for the investigation of GI illness[65] .Using the Origins Info (Experian Ltd., Nottingham ,UK) software to identify the individuals' cultural, ethnic and linguistic origins when applied to the personal and family name of the patient. Selective culture and PCR/DNA sequencing was used to identify $bla_{CTX\text{-}M}$ genogroups and types. Eighty CTX-M producing isolates were identified from 723 patients. Carriage rates in those of European origin was 8.1% and for those of Middle East and South Asia origin 22.8%,who also carried a statistically significantly higher proportion of CTX-M 15 producers[65].

A much larger, carefully stratified studied of CTX-M carriage in Enterobacteriacae across England was then undertaken[66] . Faeces from healthy individuals in the community were obtained from 2430 individuals from 4 distinct geographic locations together with a detailed questionnaire about their lifestyle and medical history. Marked geographic variation was seen. The overall rate of carriage was 7.3% but the results for individual locations were as follows: Shrewsbury 4.9%, Southampton 9.2%, Newham (London) 12.7% and Birmingham 16.0%. Particularly high rates were seen in those born in South Asia (25.0%) and in travellers in that region in the last year (38.5%). The authors suggested that patients presenting with sepsis with these risk factors should be treated with antibiotics active against ESBLs, which is supported by recent UK national guidance [67].

THE ROLE OF FOOD ANIMALS IN ANTIMICROBIAL RESISTANCE

Food is the major vector for the transmission of antibiotic resistant gastrointestinal pathogens and in the case of *Salmonella enterica* producing CTX-M β-lactamases particularly from poultry the genotypes encountered sometimes correspond with the locally common human genotypes [68]. In areas of the world where CTX-M β-lactamases are common the genotypes carried by commensal *E.coli* in food animals often are the same as human ones [69]. A survey of *E.coli* producing CTX-M β-lactamases isolated from UK retail chicken breasts in 2006 showed that CTX-M-2, a genotype common in Brazil but rare in the UK was found in samples from 4/10 imported Brazilian chicken breasts. Only 1/62 samples from UK produced chicken were positive for CTX-M 1, showing that UK produced chicken meat was not a major source of CTX-M-15 producing *E.coli*[70]. There has been considerable debate as to whether such foods can lead to colonization of the human gut with AMR Enterobacteriacae, it being argued that animal *E.coli* strains are poorly adapted for survival in the human gut[71]. However work by Professor M H Richmond and colleagues in the 1970s showed that antibiotic resistant *E.coli* could be acquired from normally cooked chicken particularly if antibiotics were being taken by the individual [72].

A comprehensive study of a small town in the Netherlands led by Dutch collaborators together with our group was undertaken to ascertain the distribution of CTX-M genotypes and sequence types of *E.coli* amongst food samples, patients with bacteraemia and faecal samples obtained on admission to hospital. The study[73] demonstrated that the same genotypes that were most common in the human subjects were present in the chicken and other meat and that those strains caused bacteraemia. CTX-M-1 was also the dominant (58.1%) genotype in chicken samples and the same genotype was also responsible for 28% of bacteraemias. Whilst we could not make an absolute link between the individual strains this study stimulated further studies.

A study was undertaken in Guangdong Province, China in 2010 to look at retail fish prior to consumption for both the presence of *bla* $_{\text{CTX-M}}$ and plasmid mediated quinolone resistance.[74] Relatively low numbers of *bla* $_{\text{CTX-M}}$ were found but all of the genotypes were those seen frequently in the Chinese population namely *bla* $_{\text{CTX-M-14}}$

and *bla* $_{CTX\text{-}M\text{-}79}$.,with 19/112 strains carrying ESBL genes. Very high rates of plasmid mediated quinolone resistance (PMQR) were found, in 59 of 80 strains picked from non-selective media. This was the first study to demonstrate the existence of high levels of PMQR genes in farmed fish in China as well as the presence of *bla* $_{CTX\text{-}M}$ ESBL genes.

More recently the abuse of antibiotics in fish farming has been highlighted in China [75] and globally [76]The recent recognition of plasmid mediated resistance to colistin in China presents incontrovertible evidence of the linkage between the usage of antibiotics in animals and the selection of resistance in human strains of *E.coli* carried in the gut. Gut carriage of *E.coli* has been suggested to be a route for the national and international movement of AMR genes in Enterobacteriaceae[77]. Colistin is not licensed for use in humans (nor has it been used) in China whereas substantial amounts are used in poultry, pig and fish production. A study[78], in Southern China, in 2014 showed that 28.0% of *E.coli* from chicken and 22.5% from pork carried the *mcr*-I gene conferring resistance to colistin. Despite no human usage 13 of 902 (1.4%) of clinical isolates of *E.coli* carried *mcr*-I [78]. Recent work from China shows mobilisation of *mcr*-I by ISEcp1 and ISApl1 into 10 different families of plasmids including the broad host range plasmids belonging to IncF1, IncF1b and IncFII[79]. Consequently we can, therefore, expect *mcr*-1 to become widely distributed and, if associated with other antibiotic resistance genes, co-selected by other agents use thus compromising one of the few remaining agents for use against AMR Enterobacteriacae.

AMR GENES FROM MAN AND ANIMALS IN THE ENVIRONMENT

Antibiotic resistant Enterobacteriacae are different from other AMR bacteria because of their unique ecology. Unlike other resistant bacteria such as MRSA, *Acinetobacter* spp, *Streptococcus pneumoniae,* AMR Enterobacteriaceae pass from our faeces into the environment in large numbers where they can persist and migrate into different parts of the ecosystem only to return to colonize and infect humans. This all occurs on a global scale, particularly with the increased in movement of people, animal and food around the world. We therefore have a circular amplification pathway with antimicrobial use causing selection at different parts of this resistome cycle driving resistance levels upwards, as depicted in figure 3. This

encapsulates the wider impact of my interest in the gut resistomes in humans and animals which is summarised in a recent review [80]. In developed countries human sewage is processed in waste water treatment plants (WWTPs). Working with colleagues at the University of Warwick, we have examined the impact WWTPs have on the resistome cycle. In a study to estimate the occurrence of CTX-M producing Enterobacteriacae in the river sediment upstream and downstream of the WWTP in Coventry we demonstrated a dramatic increase in *bla* $_{\text{CTX-M-15}}$ downstream of the WWTP. Ten novel genetic contexts for the gene were identified in Enterobacteriacae including *E.coli* ST131 and indigenous aquatic bacteria such as *Aeromonas media* [81]. Further work on the site has demonstrated that class I integrons were widely distributed together with ARM genes in both human derived strains and aquatic bacteria. We also found high boron levels (a detergent marker) downstream of the WWTP and observed that 75% of CL1s had intact QAC genes conferring resistance. Quaternary ammonium compounds (QACs)which are widely used and pass into sewage. Laboratory experiments showed successful transfer of AMR genes from these strains using QAC as the sole selective agent[82] .
This raises the question as to what the impact this route has on AMR in humans. As yet we do not have clear data on these ecosystems and further research is needed[80] . However, a recent study of people exposed to bathing waters had a higher rate of colonization with CTX-M producing *E.coli* than controls, 6.3% versus 1.5% (risk ratio 4.09, p=0.05)[83] .

CONCLUSIONS AND FUTURE PROSPECTS

AMR in Gram negative bacilli is very similar to climate change in that both are the result of human activity, both have a global impact and likely interventions will need to be made on a massive scale without results becoming immediately apparent or even being certain of success.
The development of new antimicrobial agents has been the mainstay of ensuring effective treatment of infections caused by Enterobacteriacae. The succession of agents described in this lecture from aminopenicillins to aminoglycosides, extended spectrum cephalosporins, carbapenems, polymxins and now diaza-bicyclo-octane β-lactamase inhibitors is driven by the successive spread of resistance to each class. The rate of emergence of resistance to an agent is highly unpredictable and picking

which gene(s) are going to be successful is almost impossible. The rise of different carbapenemase genes following the widespread use of carbapenems to treat infections caused by ESBL producing Enterobacteriacae is well documented with the KPC ,NDM and OXA enzymes being most common currently [77]. When carbapenems were first used it was the IMP metallo-*β*-lactamases which were most common, particularly in Japan. The first carbapenemase genotype to be described in China was IMP-4 in a single isolate of *Citrobacter* in 1998 [84] and would have been expected to spread. Initially IMP-4 was absent from surveys of Enterobacteriacae for many years, before appearing in Australia in 2004 where it spread rapidly in different species and was associated with different mobile elements to become the most important carbapenemase in Australia[85] . Recently, whole genome sequencing of the original plasmid from China showed it to have a completely different evolutionary origin to those seen in Australia, an unexpected finding [86]. The interventions that are likely to have an effect are not particularly novel but require implementation across the globe. The disparity in the size of reservoirs of AMR Enterobacteriacae is driven by high levels of human usage of antibiotics, so the implementation of good practice for treatment and infection control recently published for the UK are to be commended [8767]. Translating those for use in other parts of the world is difficult but not impossible. Action by highly influential countries which is then scaled up has been suggested to be a feasible model to achieve global action over AMR [88]. The UK's actions through the G7 and other world fora together with continuing leadership in this area is very important. China has, through national surveillance, shown that *E.coli* bacteraemia caused by CTX-M producing ESBLs has reached very high levels of 55% of those acquired in the community [89] .There is a determination in government in China to address AMR. A recent analysis has identified barriers such as perverse incentives to prescribing (i.e. linking prescribing to income) as well as poor prescribing practices. The review also suggested policy changes which, if implemented, could greatly improve the situation [90]. Possibly the biggest problem, and if solved the biggest opportunity for success, is in dealing with the agricultural use of antibiotics. Agricultural systems for food production particularly in India and China have become industrialised and globalised which at the moment demands the extensive use of antibiotics. The rise of the use of supermarkets globally as personal incomes rise[91] has had and will continue to drive

the increased rise in the industrial production of food which will have to be addressed.

As molecular microbiologists we now have the tools to investigate the multiple genetic events occurring in the gut which are involved in the spread of antibiotic resistance. A recent study of the transfer of *bla* $_{CTX-M-1}$ in strains of *E.coli* in a patient receiving multiple antibiotics revealed a dramatic diversity of strain/plasmid combinations[92] .Clearly the relationship that bacteria have with their resident R plasmids and they with its host bacterium is unique and was shown by a recent study that the fitness cost for plasmid carriage varies according to the host bacterium[93] .

Antibiotics have been widely used in large amounts for the last 70 years, but we have witnessed a plethora of genes emerge to encode resistance to each new antibiotic active against the Enterobacteriacae. Moreover the core plasmids carrying these genes appear to be capable of evolving and staying at the forefront of the selection battle. Indeed the plasmid carried by a *K.pneumoniae* strain from China encoding *bla* $_{CTX-M-14}$ originated from R100, a plasmid identified in the 1960s [94].

We have the tools at our disposal to understand the biology of AMR Enterobacteriacae and have sufficient knowledge of the interventions likely to reduce AMR. The only real question remaining is whether we have the political will to act to sustain effective treatment for patients suffering from infections due to this important group of bacteria.

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No.

Isolates

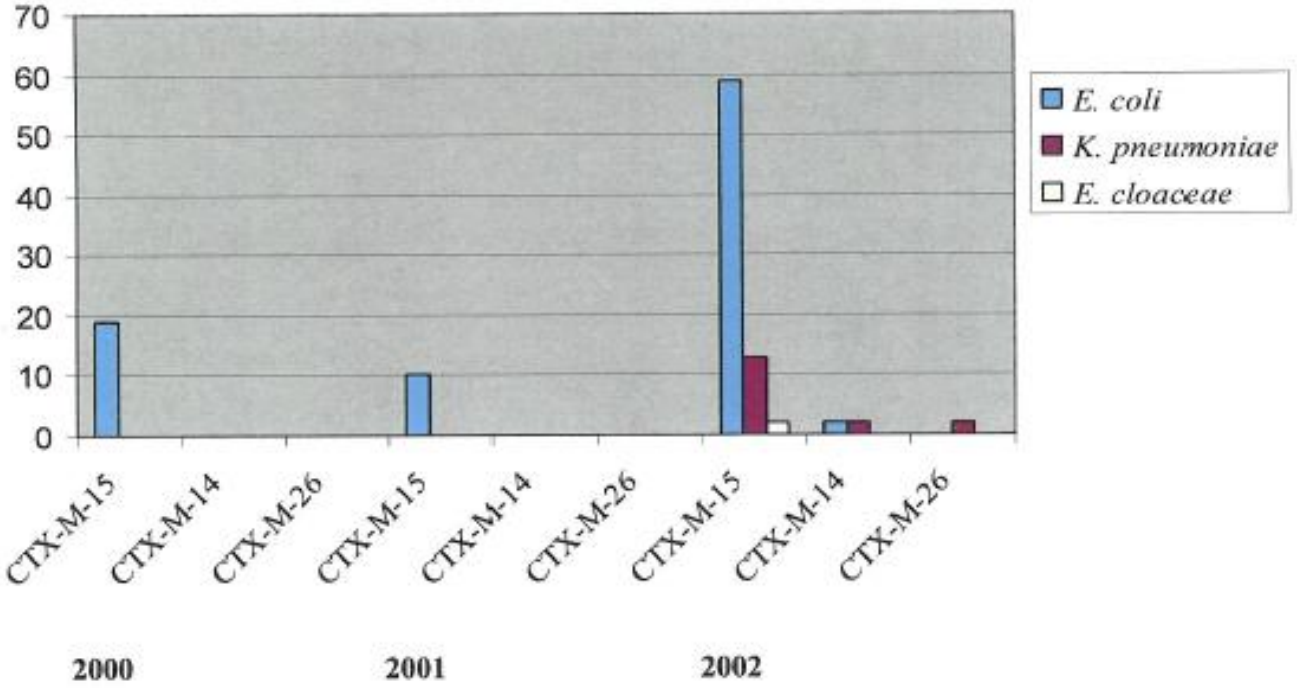

Figure 1 Occurrence of CTX-M producing Enterobacteriaceae and genotype isolated at the Queen Elizabeth Hospital 2000 – 2002 [48].

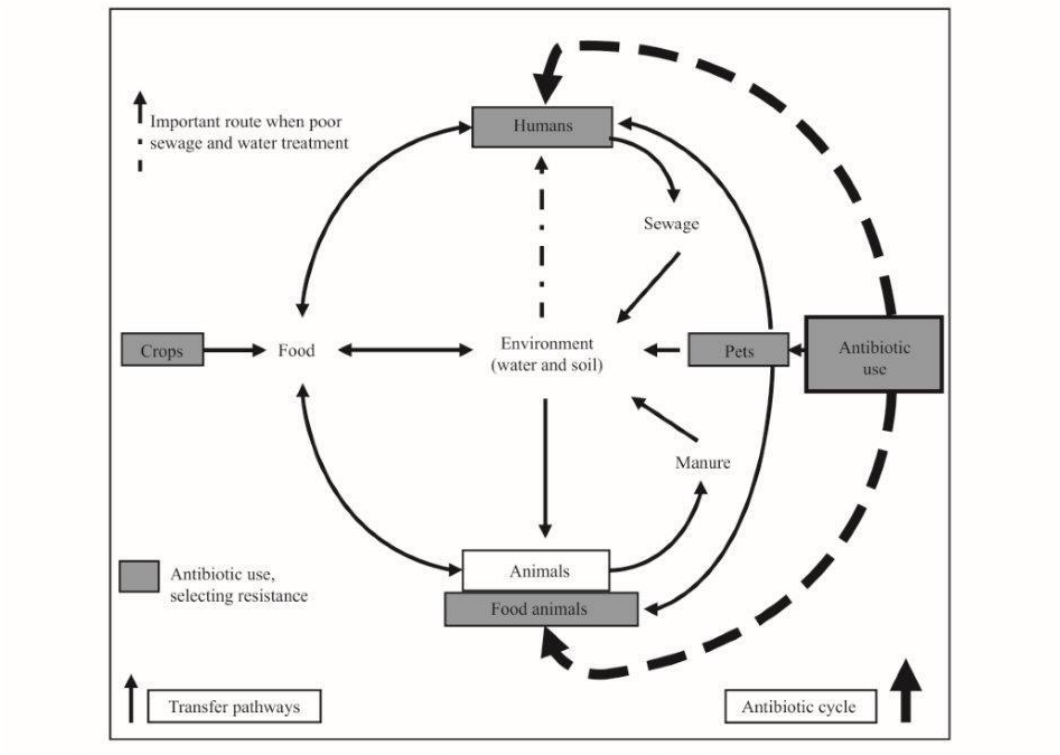

Figure 3 The resistome cycle-diagram outlining the transfer pathways for antibiotic resistance genes/bacteria between humans,animals,food and the environment[95]

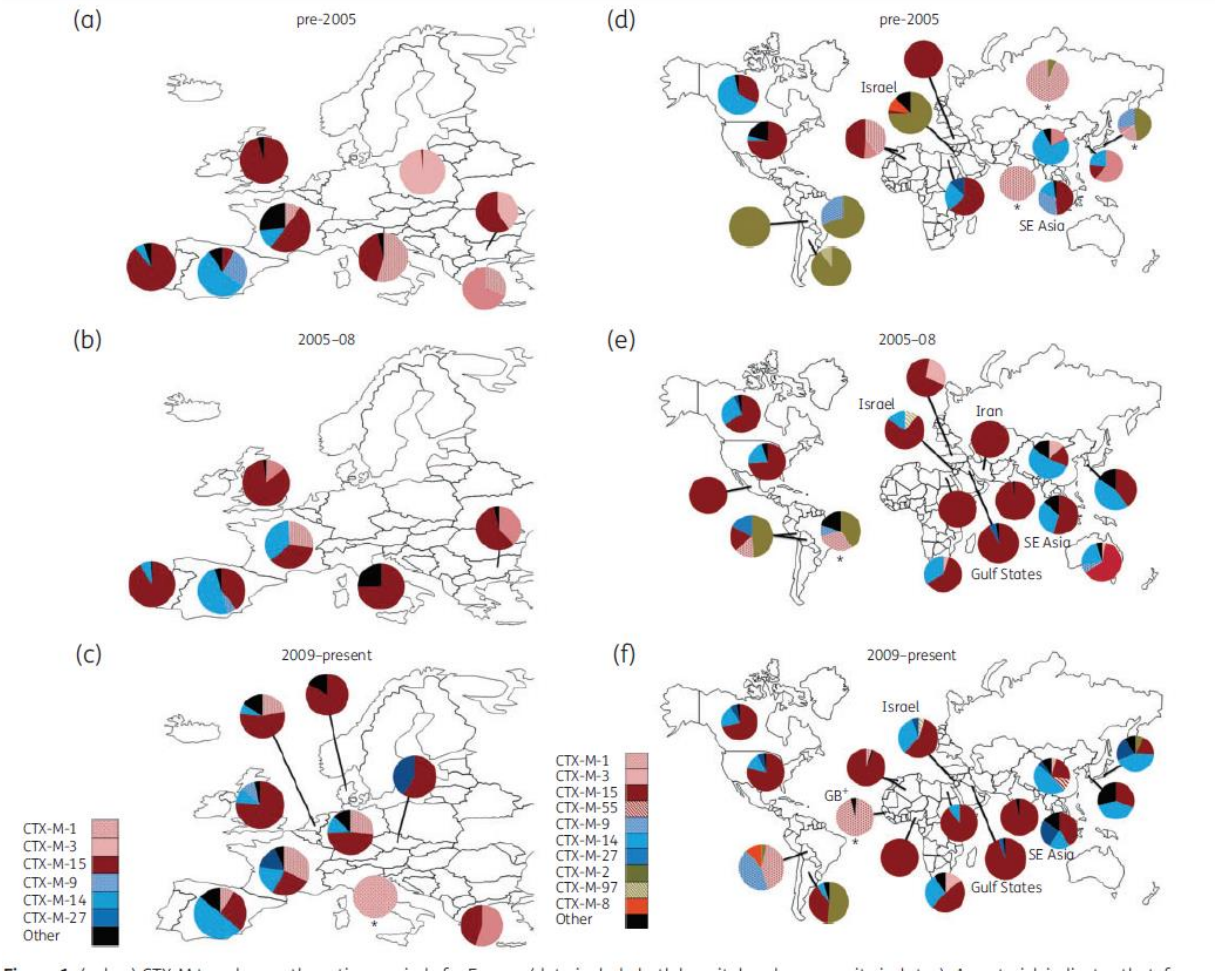

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Figure 2 Proportions of genotypes of CTX-M producing Enterobacteriacae from country studies ,* indicates only genotype group determined[58].